



Controls on soil organic carbon stability and temperature sensitivity with increased aboveground litter input in deciduous forests of different forest ages



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ABSTRACT

A previous 5-year long field litter manipulation study at the Smithsonian Environmental Research Center (SERC) in coastal Maryland demonstrated that forest age controls the chemical trajectory of litter decay and the extent and source of litter incorporation into soil physical fractions among young (60–74 yrs) and old (113–132 yrs) successional stands. To investigate if these ecosystem-level differences influence soil organic carbon (SOC) stability and temperature sensitivity, and to infer differences in stabilization mechanisms, a six-month laboratory incubation (15 °C and 25 °C) of soils from the experimental plots was conducted. The results showed that: 1) C mineralization of wood amended soils was lower than control soils in all forests with young and old forests exhibiting distinct, early vs. late, CO₂ efflux profiles over the time course of the incubation; 2) Soils from leaf-amended old forests exhibited a proportional increase in their active SOC pool but with shorter mean residence times (MRT) and a decrease in slow pools with longer MRTs, while SOC of young forests proportionally shifted to more slow cycling SOC pools with MRTs that were unchanged from controls. Structural equation modeling combining previous field and soil property data with laboratory incubation results indicated that temperature sensitivity of the active SOC pool was related to the microbial community and lignin content, while temperature sensitivity of the slow pool was related to chemical protection from silts and clays, environmental factors like pH, and soil C/N ratio. Our results underscore how successional forests of differing age can exhibit dramatically different controls on SOC-litter dynamics, through the protection and accessibility of C, that must be taken into account when predicting forest ecosystem response to future climate change.

1. Introduction

Forests in the eastern U.S. are a complex patchwork of different-age successional stands regrown from past agricultural lands abandoned since the 19th century. These ecosystems make up a large part of U.S. carbon (C) accumulation and C sink (Houghton et al., 1999). As these forests age, the evolving biogeochemical differences, such as litter chemistry and input rate, that distinguish these forest stands may influence C pool dynamics and future climate response with unknown implications for long-term terrestrial ecosystem carbon dynamics in the eastern U.S. (Liu et al., 2005; Norby et al., 2005; Pregitzer et al., 2008).

Litter, as one of the primary sources of soil organic matter (SOM), is decomposed and incorporated into soils as a result of microbial and soil

fauna activities. Litter fragments and their transformation products are incorporated into the soil and potentially protected by physical inclusion into soil aggregates or physicochemical association with the soil mineral matrix to form stable SOM (Sollins et al., 1996; Cotrufo et al., 2013; Lehmann and Kleber, 2015). Litter quantity and chemistry change during forest regrowth after abandonment and may be further altered by rising atmospheric CO₂ concentrations and increasing nitrogen (N) deposition (Fontaine et al., 2004 & 2007; Sulzman et al., 2005; Fekete et al., 2014).

The changing quantity and chemistry of aboveground litter in these developing forests may also lead to shifts of soil organic carbon (SOC) storage and stability. In addition to SOM pool size and stability, predicting the responses of the soil subsystem to rising temperatures is also highly uncertain (Friedlingstein et al., 2006). In contrast to the

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traditional “carbon-quality-temperature theory” (Bosatta and Ågren, 1999; Billings and Ballantyne, 2013), recent evidence has shown that the response of SOC decomposition to temperature change is intimately linked to the dominant SOM protection mechanisms that control the accessibility of SOM and mediate the response of the microbial community (Giardina and Ryan, 2000; Conant et al., 2011; Dungait et al., 2012). Through shifts in the interactions of substrate chemistry and accessibility, as well as the microbial community and metabolic processes, warming has considerable potential to alter the response of soils to additional litter inputs (Dijkstra et al., 2011; Cotrufo et al., 2013).

The type and length of previous land use leaves a long-lasting legacy in the soil from soil structure to belowground soil biota (Ma et al., 2013 & 2014). Among the many ecological factors that distinguish forests of different ages and successional stages in the mid-Atlantic, are distinct communities of soil ecosystem engineers, such as earthworms (Csuzdi and Szlavecz, 1999; Szlavecz et al. 2018). Earthworm community composition has been directly correlated with rates of litter transformation and soil C dynamics in the Mid-Atlantic area (Crow et al., 2009a; Yesilonis et al., 2016; Szlavecz et al., 2018). Previous studies have demonstrated that leaf feeding, epigaeic earthworms may directly alter the forest floor, while soil feeding endogeic earthworms largely control the quantity of litter incorporated into mineral soils and soil aggregation dynamics through feeding and burrowing (Jégo et al., 1998a,b; Lubbers et al., 2017). How these many changing factors, especially changing earthworm abundance and species composition, during forest redevelopment in mid-Atlantic area may affect responses of SOC to future ecosystem changes, is largely unknown (Lytle et al., 2015; Zhang et al., 2016; Szlavecz et al., 2018).

The forest stands at the Smithsonian Environmental Research Center (SERC), USA, represent a complex patchwork of mixed age forest stands with well-documented land use history and plant composition, as well as associated gradients of soil properties, invertebrate community composition and activity (Filley et al., 2008; Ma et al., 2013; Yesilonis et al., 2016). Previous research at SERC using long-term field manipulation of increased litter input and varied litter types (e.g. leaf tissue vs. woody tissue) demonstrated that the chemical trajectory of litter decay, and the dynamics of its incorporation and distribution among physical fractions in surface soil was distinct between young (60–74 yrs) and old (113–132 yrs) successional forest stands (Filley et al., 2008; Crow et al., 2009a; Ma et al., 2013). Specifically, in young forests relatively more litter C was incorporated into aggregates and mineral-associated fractions (Ma et al., 2014), while in old forests, the newly incorporated C was relegated to particulate organic matter (POM).

To quantify the consequences of observed changes in soil physical and chemical properties on the stability, microbial accessibility and temperature sensitivity of SOC pools, a laboratory incubation experiment at two temperatures (15 °C and 25 °C) was conducted, using soils collected after 5 years of increased tulip poplar (*Liriodendron tulipifera*) leaf and wood litter input in young and old forest stands at SERC. It was hypothesized that young forests, where newly incorporated aboveground litter C was associated with mineral surfaces and within soil aggregates (Ma et al., 2014), would exhibit an increased stable C pool with low temperature sensitivity. Older successional forests, where newly incorporated aboveground litter C was relegated more to POM, would exhibit an increase in the active C pool with greater temperature sensitivity. In addition, soil properties observed in the field litter manipulation study and SOC pool results from laboratory incubations were incorporated into a structural equation model (SEM) to tease apart the controlling soil properties on SOC stability and temperature sensitivity at SERC forests.

2. Methods

2.1. Site description

The litter manipulation experiments were set in the forests of the Smithsonian Environmental Research Center (SERC), (38°53' N, 76°33'

W), Maryland, USA. This area has long been impacted by humans, particularly by European colonists, until being abandoned since late 1800's. The research sites were in the region of tulip poplar association, in which young forests (60–74 yrs) are dominated by tulip poplar (*Liriodendron tulipifera* L.) and sweet gum (*Liquidambar styraciflua* L.) trees and old successional forests (113–132 yrs) are dominated by tulip poplar alone, with white oak, red/black oaks, American beech, and hickories (*Carya* spp.) as the secondary tree species (G. Parker, unpublished data). Soils of this area were classified as Collington sandy loam (fine-loamy mixed, active, mesic Typic Hapludult) and Monmouth fine sandy loam (fine, mixed, active, mesic Typic Hapludult) with similar texture and mineralogy (Pierce, 1974).

Earthworm community surveys at SERC have been conducted for nearly two decades (Szlavecz and Csuzdi, 2007; Szlavecz et al., 2018). To date, a total fourteen species have been recorded from this experimental forest stands, eight of which were regularly collected (Szlavecz et al., 2018). Earthworm density varied between 25 ind m⁻² and 467 ind m⁻² depending on site, season and year. In general, earthworm density was higher in young than in old forests, while biomass was not significantly different between the two types. The main distinction between stands of different ages and successional stages is in species and functional group composition and relative abundance of functional groups: both absolute and relative abundance of endogeic earthworms was higher in the young than in the old forests (Table S1).

2.2. Soil sampling and storage

Briefly, five litter manipulation experimental sites (two old successional forests and three young successional forests) were established in 2003. Each plot contained six replicates of either chipped tulip poplar wood or crushed tulip poplar leaves, randomly distributed within the grid of 36 subplots. Leaf litter collected in the previous year was added to each ringed subplot twice (spring and fall) annually, corresponding to approximately 2.5x (leaf treatment) mean annual leaf fall. Chipped tulip poplar wood (wood treatment) was added in 2003, 2004 and 2006 at a rate of 100 g per subplot for each addition (about 10x average estimates of background coarse woody debris (CWD) input). Nylon mesh with 1.25 cm hole size was used to cover the plot and capture autumn leaf fall, which was added back to the control plots during the year.

Six soil cores (0–5 cm deep, 5 cm diameter) were collected from each treatment; an additional six randomly chosen control cores were taken from soil surrounding the plot. The six cores were reduced to three composite cores by randomly choosing three sets of two cores to combine within each plot. Field-moist soils were passed through 8 mm, followed by 4 mm and 2 mm, sieves to remove rocks, large roots, and other debris. The sieved soil samples were air dried until constant weight and then stored in glass jars until further processing. Soils from each treatment (leaf, wood, control) at each site were pooled together and used in the incubation study presented here.

2.3. Soil characteristics with field litter amendments

As recently described in detail (Ma et al., 2014), among five SERC forest sites, old forests (113–132 yrs) had higher C, N, and substituted fatty acid (SFA) concentrations than young forests (60–74 yrs), but had lower soil pH and lignin/SFA ratio (Table S2). After five years of increased litter amendments, the SOC content, pH, and biopolymer chemical distributions among soil physical fractions in the top 5 cm of soils were altered, but this effect was primarily in young forests and subplots with wood amendments. Specifically, wood amendments in young forest soils resulted in an increase of SOC, C/N ratio, and decrease in soil pH (Table S2). The increase in soil C with wood addition was found to mainly POM, which included coarse POM (cPOM) and inter-microaggregate POM (iPOM) fractions (Fig. S1). In contrast, bulk C content and C/N ratio in old forests exhibited only a modest change with wood

addition, compared to young forests. With leaf amendments in old forest soils, no significant effect was observed in either C or N concentration or in the physical distribution of chemical parameters among soil particles.

Lignin and SFA chemistry were used previously to track the incorporation of aboveground litter amendments to bulk soil. With wood addition, lignin concentration in the top 5 cm of soils increased approximately 70% in young forests and 30% in old forests. Wood addition also resulted in a quantitative decrease in SFA. However, with amendments of almost 2.5 times background leaf input, neither lignin nor SFA concentration changed in the young or old forests (Table S2).

2.4. Microcosm respiration measurements

Soils (0–5 cm) were incubated in 12 mL vials with septa caps (Labco, UK). The bottoms of the vials were packed with approximately 0.5 cm of ashed glass wool to limit water accumulation and prevent anaerobic conditions. Air-dried soil (1.5 g) and ashed quartz sand were mixed and added to each vial at a 1:1 ratio to increase aeration during the incubation. The experiment was initiated after wetting the soil to 60% water holding capacity (WHC) with a microbial inoculum that was prepared from a composite of fresh soil samples taken immediately adjacent to the plots at each site. To create the inoculum, the composite soil was mixed with distilled water at 1:10 (soil: water) mass ratio, shaken for 1 h, then filtered through an ashed Whatman GF/F glass filter paper (pore size 0.7 mm) (Crow et al., 2006; Creamer et al., 2011).

The microcosms were incubated in the dark at two temperatures: 15 °C and 25 °C. 60% WHC was maintained by periodic weighing and addition of small amounts (< 200 µL) of sterile water. The quantity of CO₂ respired by soil microorganisms was determined directly from the microcosm vials on days 1, 3, 5, 7, 10, 14, 21, 28, and subsequently every 28 days for six months after initiation of the experiment. CO₂ was analyzed using a Varian GC system (Agilent Technology, Santa Clara, US).

Prior to each sampling event, vials were flushed with 25x their volume of humidified, CO₂-free air created by passing atmospheric air through an indicating NaOH filled trap then bubbling through sterile water. CO₂ respired by microorganisms was then allowed to accumulate in vials for subsequent measurement on the GC. For the first week of the incubation, CO₂ accumulated for 6–24 h. The accumulation time for 15 °C was generally longer than 25 °C due to lower respiration rate. After one month, the vials needed to accumulate CO₂ for up to 7 days to obtain measurable CO₂ concentrations. When not accumulating CO₂, the caps with septa were replaced with GF/F filters to permit normal gas exchange. After the last sampling day, vials were kept frozen at –80 °C prior to freeze-drying and microbial analysis (see below).

2.5. Fungal and bacterial abundance measurement

To investigate the change in the microbial community during the incubation period, one set of vials (three replicates for each treatment) was harvested after one month and one set after six months of incubation. All soil samples were freeze-dried and then ground to homogenize samples. The relative abundance of fungal and bacterial nuclear ribosomal DNA in the soils was assessed using quantitative real-time polymerase chain reactions (qPCR). DNA was extracted from two 250 mg subsamples of soil from each microcosm using PowerSoil DNA extraction kits (Mo Bio Inc., Carlsbad, USA). After extraction, total DNA was quantified using a NanoDrop-2000 microvolume spectrophotometer (Thermal Scientific, Wilmington, USA). 10 ng of DNA from each sample was amplified using qPCR with bacterial and fungal specific PCR primers (specified below) to determine the relative abundance of DNA from different microbial taxa. The ratio of fungal: bacterial DNA (F/B ratio) was calculated to determine how the relative abundances of these taxa changed as a result of litter manipulations.

qPCR reactions were conducted in 96-well plates with an MJ Research Opticon DNA Engine with Continuous Fluorescence Detection

(MJ Research, now Bio-Rad Laboratories, Hercules, CA, USA). For fungal abundances, each 25 µL reaction contained 12.5 µL iQ SYBR Green PCR Super Mix (Bio-Rad Laboratories, Hercules, CA, USA), 10 ng DNA template in 10 µL H₂O, and 1.25 µL (10 mM) of each of the primers ITS1F and 5.8S (Fierer et al., 2005). QPCR amplifications were conducted as follows: initial denaturation at 95 °C for 5 min, followed by 41 cycles of 15 s denaturation at 94 °C, 30 s annealing at 53 °C, and 30 s elongation at 72 °C, followed by a plate read step. The same method was used to determine bacterial relative abundance, but with the primers Eub338 and Eub518 (Fierer et al., 2005) and qPCR parameters as follows: 95 °C initial denaturation step for 5 min followed by 41 cycles of 15 s denaturation at 94 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C. Each sample extract was amplified in triplicate, and quantified based on the critical threshold intercept. PCR product from the target region in a *Tomentella* sp. (isolate #M259) and *E. coli* were used to construct standard curves for fungi and bacteria, respectively. A melting curve analysis was performed after each analysis to confirm the specificity of the qPCR.

2.6. Data analysis

Although SOM production/destruction processes (e.g. litter decay, mineral adhesion and exchange, aggregation, microbial biomass production) have been effectively articulated as a continuum of processes (Lehmann and Kleber, 2015), conceptual pool modeling has been used to help quantify shifts in C stability and mean residence time (MRT), and provide information related to aggregated C protection mechanisms. Cumulative CO₂ mineralization profiles were used to determine the turnover rates and sizes for the active (Ca) and slow (Cs) carbon turnover pools using the two-pool model developed by Paul et al. (2001):

$$C_{\text{total}} - C_{\text{respired-}t} = C_a \cdot e^{-k_a t} + C_s \cdot e^{-k_s t}$$

where C_{total} is the percentage of total initial C content, which is always expressed as 100%, $C_{\text{respired-}t}$ is the cumulative percent of total SOC respired at time t , C_a is the size of the active pool (expressed as a percentage of total SOC), k_a is the turnover rate of the active pool, and C_s and k_s are the size and turnover rates of the slow pool. The percentage of the total initial C content was used here instead of the absolute amount of C to study changes in C stability and turnover caused by litter addition rather than changes in absolute C storage. The mean residence times (MRT) of the two pools were determined by taking the inverse of the turnover rates (1/k). The size and MRT of the resistant pool (Paul et al., 2001) were not determined separately, therefore C_s should be interpreted as the resistant pool and the slow pool combined. The size of the slow pool (C_s) was determined as the difference between total SOC and C_a . Without including the resistant pool, the two-pool model can underestimate the size and MRT of C_s , but the size and the MRT of C_a is unchanged (Paul et al., 2001).

The model parameter determination was conducted using the non-linear curve fitting function "lsqcurvefit" in MATLAB (version R2012a). A three-way ANOVA analysis, using the "GLM" function in SAS 9.3, was performed to determine treatment effects of incubation temperature, amendment type and forest age and their interactive effects for leaf and wood litter type individually. Differences in respired C per gram SOC, C pool sizes, as well as MRTs between litter amended soils (either wood or leaf treatment) and control soils were tested with student *t*-tests for all sampling points within each age group for 15 °C and 25 °C respectively. Bonferroni corrections for multiple comparisons were applied to control family-wise error rate, thus for each comparison, $\alpha = 0.025$ was set for significant difference and $\alpha = 0.05$ was set for marginal significant difference.

In addition, we used structural equation modeling (SEM) to investigate how possible direct and indirect effects of soil properties on soil C stability and temperature sensitivity were mediated by microbial

communities. SEM has been widely used in social sciences to test hypotheses inferred from causal diagrams under a graph theory framework, and recently adapted to study complex systems in soil ecology (Grace et al., 2012; Sackett et al., 2013; Eisenhauer et al., 2015). With particular relevance to this study, SEM has recently been used to test species effects of earthworms on soil and microbial properties at SERC (Chang et al., 2016). In the current study, we used previously published geochemical properties from soils at these sites (Ma et al., 2013, 2014), including organic C and N content, C/N ratio, soil pH, C proportion inside microaggregates (mAG: iPOM + iSC), C proportion associated with silt and clays (SC: fSC + iSC), as well as lignin and substituted fatty acid (SFA) concentration, for the modeling. Soil microbial communities were represented using the fungi: bacteria ratio measured by qPCR. Size and MRTs of C pools were used to represent C stability. Temperature sensitivity of the active and slow pools was represented using MRTs of active or slow pools at 25 °C compared to 15 °C. The model was established and run in IBM SPSS + AMOS 22.0. Regression parameters were estimated using the Maximum Likelihood estimation method. Direct and indirect effects were estimated using bootstrap analysis.

3. Results

3.1. C mineralization during incubation

After 6 months of incubation at 15 °C, old successional forest soils lost 57.14 ± 6.50 , 53.34 ± 9.24 and 57.00 ± 7.59 mg C per gram SOC for control, leaf and wood amended soils, respectively (Fig. S2), while young forest soils lost 44.07 ± 1.84 , 40.01 ± 3.53 and 41.73 ± 2.15 mg C per gram SOC for control, leaf and wood amended soils respectively. At 25 °C, old successional forest soils lost 96.56 ± 10.78 , 87.24 ± 6.54 and 100.12 ± 16.99 mg C per gram SOC for control, leaf and wood treatments respectively, while young forests lost 74.89 ± 4.10 , 69.77 ± 7.24 and 70.53 ± 4.16 mg C per gram SOC for control, leaf and wood treatments, respectively, after 6 months. Within both forest age groups, the respiration rate was much higher in the first two weeks of the experiment than for the remainder, resulting in 45–50% of the total CO₂ loss occurring in this period in young forest soils.

Litter amendments significantly influenced respiration patterns in both young and old forest soils, but in different ways (Fig. 1, Figs. S2; Table S3). At 15 °C, leaf-amended old forest soils respired proportionately more C at the beginning of incubations than other treatments did, but this increased C mineralization diminished after day 56. The greatest difference was exhibited in the first week of the experiment, e.g. respiration rates at day 2 were 220.8 ± 20.4 , 431.6 ± 4.1 , 189.3 ± 25.2 µg C per gram organic carbon respired/h for control, leaf and wood amended soils, respectively (Table S4). At the end of 6-month incubation, the respiration rate of control, leaf and wood amended soils were 11.1 ± 4.7 , 6.3 ± 0.9 , 11.2 ± 5.4 g C/h µg C per gram organic carbon respired/h (Table S4). In contrast, SOC mineralization in young successional forests was lower in leaf-amended soils than in control soils during the entire incubation time. Wood amended old forest soils also respired less C than control soils until day 84. In young forests, however, wood amended soils did not exhibit slower respiration until the very end of incubation, at day 168. At 25 °C, the overall pattern of the influence of litter amendments was similar to that found at 15 °C, only with a faster transition of changes through time. For example, the suppression of C mineralization under wood amendments that was seen in old forest soils started to decrease much earlier (day 14) at 25 °C compared to 15 °C (day 84) (Fig. 1; Table S3).

3.2. Carbon pool sizes and MRTs

Results from the two-pool modeling of proportional C respiration in the laboratory incubation showed interactive effects of litter type, forest age, and incubation temperature on calculated C pool sizes and their

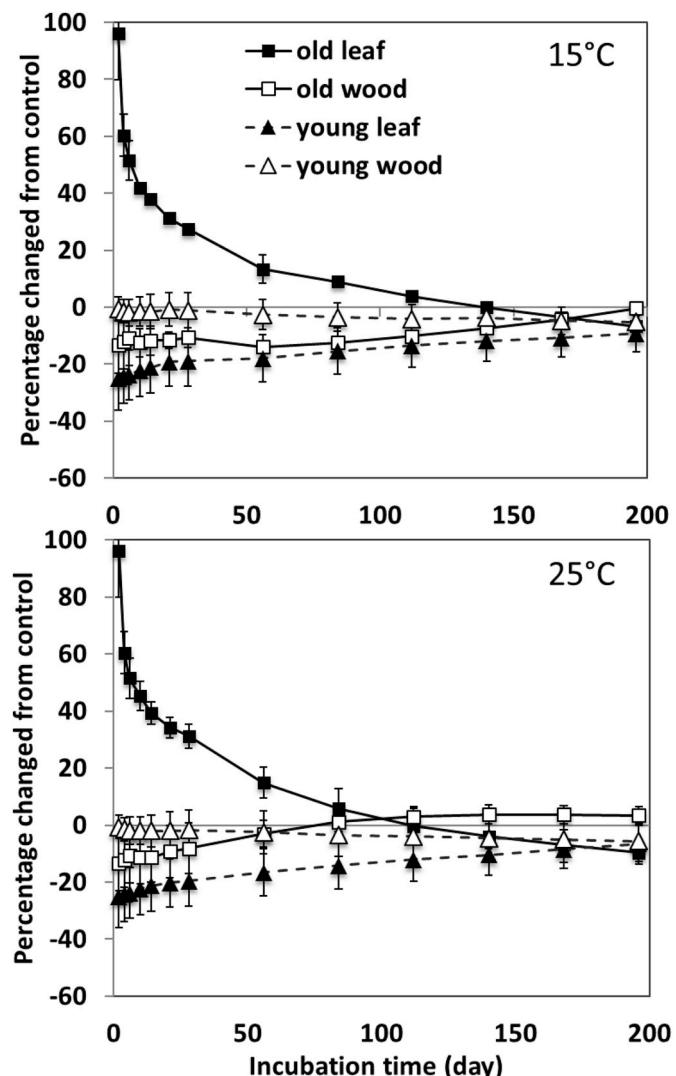


Fig. 1. Percentage changes of cumulative CO₂ respiration per gram organic carbon in response to leaf or wood litter treatment, compared to control, over 6 months of incubation at 15 °C (upper panels) and 25 °C (lower panels).

MRTs (Tables 1 and 2). Overall, the active carbon pool sizes ranged from 1.17% to 2.15% at 15 °C and 2.64%–4.21% at 25 °C across all treatments (Table 1). Amendment type influenced the size of the active C pool differently in young and old forest soils. Leaf amendments significantly increased the active pool size in old forest soils, but had the opposite effect in young forests. In contrast, wood amendments decreased the active pool size in old forest soils, but had no measurable effect in young forest soils. Increasing temperature significantly increased the C active pool size for all samples with the size of the pool at 25 °C being about twice as much at 15 °C. The mean residence time (MRT) of the active pools ranged from 3.17 to 6.34 days across all treatments but with no significant difference between the two temperatures (Table 1). Leaf amendments significantly decreased the active pool MRTs at both 15 °C and 25 °C in old forest soils, while all other litter amendments exhibited no alteration of the MRT of active pools.

With only about 1–4% of SOC being in active pools, the majority of C (96–99%) remained in the slow pool, with MRT ranging from 11.69 yrs to 20.81 yrs and 6.57 yrs–13.72 yrs at 15 °C and 25 °C respectively (Table 1). The MRT of slow pool C was significantly influenced by temperature, treatment (litter amendment), forest age and the interactions of temperature*forest age and treatment*forest age (Table 1). Specifically, increasing the incubation temperature from

Table 1

Active pool size (expressed as a percentage of SOC), active pool mean residence time (MRT; expressed in days), and the slow pool MRT (expressed in years), as estimated by the two-pool model for soil incubation. Mean values are given \pm standard deviation. Asterisks (**) indicate significant changes ($P < 0.025$) and (*) indicate marginal significant changes ($P < 0.05$) of leaf or wood amended soils, compared to controls, after Bonferroni correction for multiple comparisons.

			Active pool size (% of SOC)			Active pool MRT (day)			Slow pool MRT (yr)		
15 °C	old	control	1.57	\pm	0.12	6.24	\pm	1.89	12.20	\pm	1.30
		leaf	2.15	\pm	0.04	3.55	\pm	0.44	15.35	\pm	2.38
		wood	1.17	\pm	0.06	6.34	\pm	2.23	11.69	\pm	1.35
	young	control	1.79	\pm	0.09	3.95	\pm	0.97	18.98	\pm	1.53
		leaf	1.37	\pm	0.10	4.27	\pm	1.04	19.38	\pm	1.78
		wood	1.79	\pm	0.05	4.15	\pm	1.26	20.81	\pm	1.68
25 °C	old	control	2.93	\pm	0.28	4.97	\pm	0.40	7.25	\pm	1.16
		leaf	4.21	\pm	0.10	3.17	\pm	0.27	10.49	\pm	1.95
		wood	2.64	\pm	0.12	4.34	\pm	0.82	6.57	\pm	1.09
	young	control	3.56	\pm	0.20	3.73	\pm	1.01	12.30	\pm	0.78
		leaf	2.70	\pm	0.17	3.88	\pm	0.87	11.96	\pm	1.37
		wood	3.52	\pm	0.11	3.76	\pm	1.05	13.72	\pm	1.54

Table 2

P-values from a three-way ANOVA testing the significance of effects of temperature (15C, 25C), amendment treatment (leaf, wood, control), and forest age (young, old) on active pool size, active pool MRT and slow pool MRT. Bolded values indicate significant effects ($\alpha = 0.05$).

	Active pool size	Active pool MRT	Slow pool MRT
leaf			
temp	< .0001	0.048	< .0001
treat	0.7745	0.0044	0.0002
temp*treat	0.8529	0.6179	0.671
age	< .0001	0.0473	< .0001
temp*age	0.0579	0.3242	0.0093
treat*age	< .0001	< .0001	0.0002
temp*treat*age	< .0001	0.304	0.5903
wood			
temp	< .0001	0.9139	< .0001
treat	0.0002	0.9123	0.0397
temp*treat	0.7601	0.0708	0.6523
age	< .0001	< .0001	< .0001
temp*age	< .0001	0.3247	0.0123
treat*age	< .0001	0.5706	0.0031
temp*treat*age	0.3034	0.0132	0.8676

15 °C to 25 °C decreased the MRT of slow pool C by approximately 30–40% for all soils. In addition, and in contrast to the decreased MRTs for active pools, at both 15 °C and 25 °C leaf amendments significantly increased the MRT of slow pool C in old forest soils. Similarly, wood amendments significantly increased the MRT of slow pool C in young forest soils but the extent of this change was less than the effects of leaf amendments in the old forest soils.

No significant interaction was detected for temperature*treatment on either the size or the MRTs of the two pools (Table 1), indicating that neither amendment type changed the temperature sensitivity of either C-pool. But noticeably, although not statistically significant, the active pool MRT ratio of 25 °C over 15 °C, which reflects temperature sensitivity of SOC pools, decreased with wood amendments. In young forests the MRT ratio of neither the active nor the slow pool changed with amendments (Fig. S3). A significant interaction with treatment*forest age was detected for MRT for all treatments except for the active pool with wood amendment, indicating that litter amendments altered the size and MRT of soil C pools differently depending on the age of the forest, which also co-varied with earthworm abundance and activity (Szlavicez and Csuzdi, 2007).

3.3. Bacterial and fungal DNA abundance

When incubated at 15 °C, both leaf and wood amendments triggered an increase in bacterial abundance over the 6-month incubation period

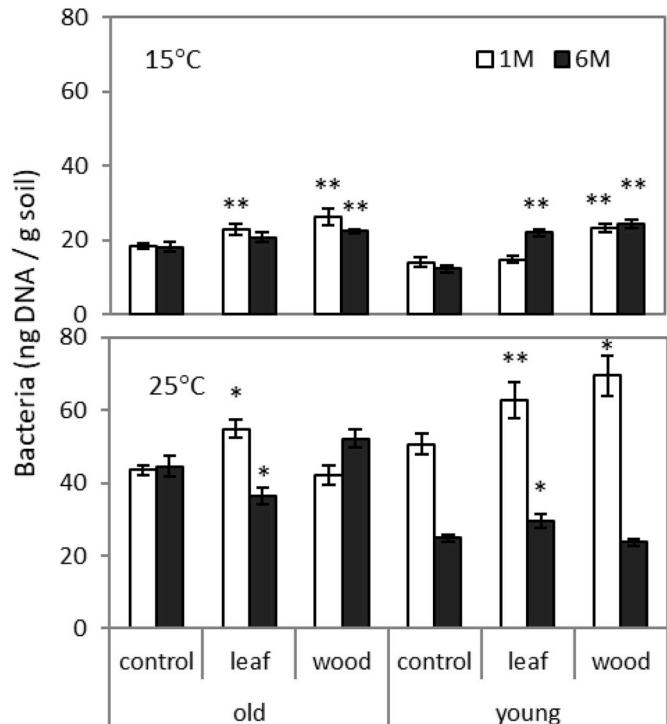


Fig. 2. Bacterial DNA concentration after 1 month (1M) and 6 month (6M) laboratory incubations of old and young forest soils with leaf and wood amendment treatments and controls at 15 °C (top) and 25 °C (bottom). Error bars indicate standard error. Asterisks (**) indicate significant changes ($P < 0.025$) and (*) indicate marginally significant changes ($P < 0.05$) of leaf or wood amended soils compared to controls after Bonferroni correction for multiple comparisons.

(Fig. 2; Table S5). At 25 °C, leaf-amended soils showed an increase in bacterial DNA at 1 month (1M) for both young and old forest soils. In wood amended soils, only young forests showed a marginally significant increase in bacterial DNA abundance at the 1M sampling point. After 6 months of incubation at 25 °C, there was a significant reduction in bacterial DNA abundance in young forest soils for all treatments. Fungal DNA abundance was significantly higher in wood amended soils at all sites, but the increase, compared to control, was much larger when incubated at 15 °C than at 25 °C (Fig. 3; Table S5). In addition, the percent increase in wood amended soils was larger in old than young forest soils. Under the higher incubation temperature (25 °C), the fungal DNA abundance dropped significantly between 1M and 6M, while it

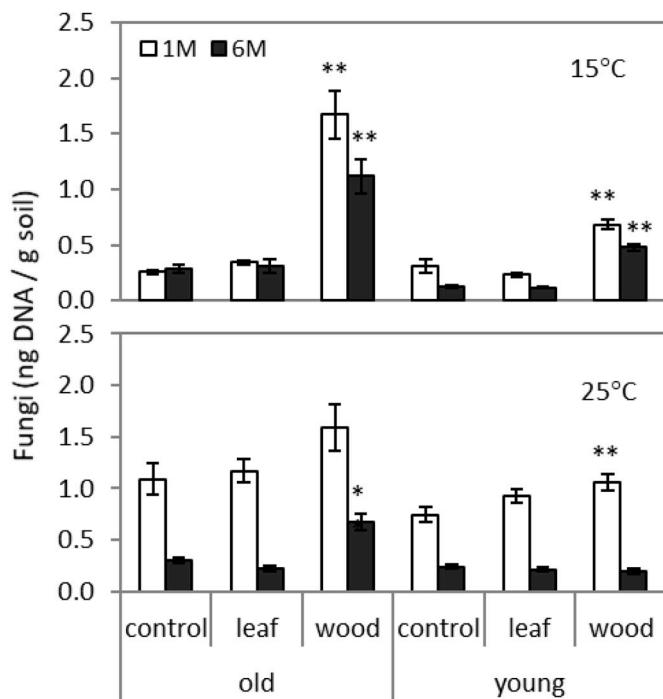


Fig. 3. Fungus DNA concentration after 1 month (1M) and 6 month (6M) laboratory incubations of old and young forest soils with leaf or wood amendment treatments and controls at 15 °C (top) and 25 °C (bottom). Error bars indicate standard error. Asterisks (**) indicate significant changes ($P < 0.025$) and (*) indicate marginal significant changes ($P < 0.05$) of leaf or wood amended soils compared to controls after Bonferroni correction for multiple comparisons.

remained unchanged over that time period when incubated at 15 °C.

Because fungal DNA abundance increased to a larger extent than bacterial DNA, the ratio of fungi/bacteria DNA abundance (F/B ratio) was significantly higher in wood amended soils from old forests, especially when incubated at 15 °C (Fig. 4; Table S5). However, in young forest soils, the higher F/B ratio of wood amended soils only occurred after 6 months of incubation at 15 °C. The overall lower F/B ratio at 25 °C indicated that at a higher temperature the soil microbial community shifted towards more bacterial dominance. Leaf amended soils did not show a F/B ratio shift compared to control soils during the 6-month incubation except for young forest soils incubated at 15 °C (Fig. 4; Table S5).

3.4. SEM results

The SEM model adequately fit the data on soil respiration ($\chi^2 = 96.46$, $df = 33$, $P < 0.01$, Fig. 5, Table 3). It explained 95.1% of the variance in MRT of slow pool, 68.3% and 88.4% of the variance in temperature sensitivity of active and slow pool C, and 82.6% of the variance in fungi: bacteria ratio. SEM results gave possible causal effects of different factors. SFA content and F/B ratio had significant direct negative effects on the MRT of slow pool C, while soil pH and mAG C proportion had direct positive effects on the MRT of slow pool C. Interestingly, no factor included in this model had any significant effect on the MRT of active pool C (P value of those paths > 0.1). The F/B ratio had a significant direct positive effect on the temperature sensitivity of active pool C. Additionally, by influencing the F/B ratio, lignin content and soil pH had marginally significant ($0.05 < P < 0.1$) indirect positive and negative effects, respectively, on the temperature sensitivity of the active pool (Table 3, Fig. 5). Soil pH had a significant direct positive effect on the temperature sensitivity of slow pool C while SC C content and soil C/N ratio had direct negative effects on the

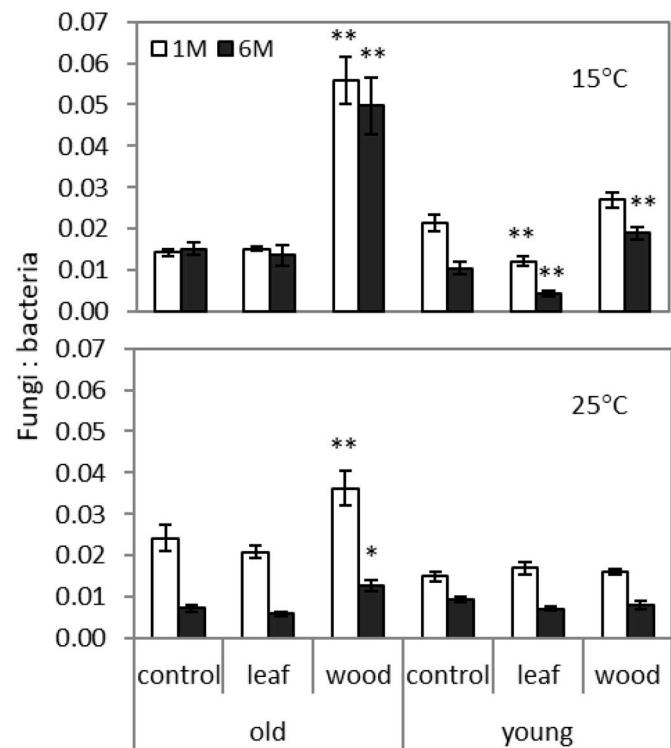


Fig. 4. Fungi:bacteria ratio after 1 month (1M) and 6 month (6M) laboratory incubations of old and young forest soils with leaf or wood amendment treatments and controls at 15 °C (top) and 25 °C (bottom). Error bars indicate standard error. Asterisks (**) indicate significant differences ($P < 0.025$) and (*) indicate marginally significant differences ($P < 0.05$) of leaf or wood amended soils compared to controls after Bonferroni correction for multiple comparisons.

temperature sensitivity of the slow pool C.

Overall, soil pH had the strongest effects on the F/B ratio and the stability and temperature sensitivity of soil C pools, while mAG carbon influenced the MRT, and SC carbon influenced the temperature sensitivity of slow pool C. All factors included in our study, except C/N ratio, directly influenced F/B ratios, and so, may indirectly influence C pool dynamics.

4. Discussion

4.1. SOC pool responses to litter addition varied with forest age

In the eastern U.S., forest carbon accumulation has been attributed to regrowth of forests after agricultural abandonment, fire suppression, and reduced fuelwood harvesting (Houghton et al., 1999; Caspersen et al., 2000; Albani et al., 2006). Previous land use, timing of abandonment, and secondary forest composition influenced not only aboveground C accumulation, but also C pool dynamics in soils. The long-term litter manipulation study at SERC demonstrated the chemical trajectory of litter decay, and the dynamics of its incorporation and distribution among physical fractions in surface soil was distinct between young and old forest stands (Filley et al., 2008; Crow et al., 2009a; Ma et al., 2013). In this study, these different responses observed in field were reflected in SOC pool dynamics with laboratory incubation. In old forest soils, C shifted to a state with a faster cycling active pool and a stabilized slow pool, while in young forest soils, the sizes of active and slow pools shifted, without significant changes of MRT for either active or slow pool (Fig. 1, Table 1). Soils in SERC forest sites with different ages differed in a number of chemical, biological, and physical properties that could account for these distinct responses to litter addition (Ma et al., 2013).

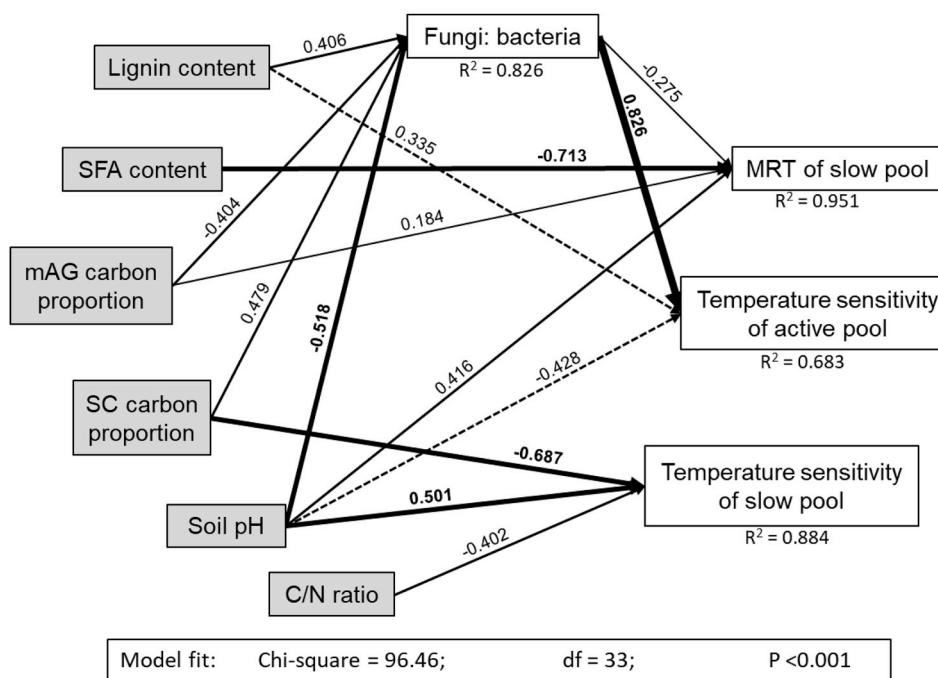


Fig. 5. Structural equation model showing predicted effects of soil properties and microbiota on carbon pool stability and temperature sensitivity and how these effects are mediated by the soil microbiota. Exogenous variables are indicated by grey rectangles and endogenous variables are indicated by white rectangles. Arrows with dashed lines indicate indirect effect pathways. Arrow thickness is scaled proportionally to the standardized path coefficients (numbers on arrows). Variances explained by the model (R^2) are shown next to each endogenous variable.

Previous research at SERC has found that although total SOC did not change, the amount of cPOM increased after double leaf litter addition in old forests (Fig. S1). This pattern of incorporation may result in more labile SOC and nutrients, but also trigger a positive priming effect – “acceleration of mineralization induced by addition of an easily decomposable energy source,” (Kuzyakov et al., 2000; Cleveland et al., 2007) that depletes native SOC pool and replaces it with fresh leaf litter C. Correspondingly, SOC in old forests shifted to a state with a faster cycling active pool and a stabilized slow pool after 5 years of leaf amendments. In young forests, the proportion of cPOM decreased indicating the decomposed. The newly incorporated litter was, however, more associated with minerals or within microaggregates, which enhanced the protection of that new litter pool and resulted in a relative shift from active SOC pool to slow pool (Table 1).

Among many ecological properties that differ among SERC forest stands, earthworm abundance and community composition may contribute considerably to the different responses of SOC pools to enhanced leaf amendments and temperature. The 19-year soil invertebrate

monitoring program at SERC forests has demonstrated that forests of different ages have distinct earthworm communities as a result of environmental filtering through leaf litter quality (Szlavecz et al., 2018). Specifically, soil feeding species that rely on relatively fresh SOM derived from fast-decomposing litter are more abundant in young than in old forests. Endogeic earthworms in old forests are also functionally different; they feed on highly processed SOM derived from slow-decomposing litter. Additionally, the relative abundance of litter feeding earthworms is higher in old forests than in young forests (Table S1).

The higher proportion of leaf feeding earthworms in old forests at SERC should increase the incorporation of fresh litter C and more available nutrients into surface soils causing an “*in-situ*” priming effect and subsequent shift of SOC pools. An “*in-situ*” priming effect was evoked as response in the Detrital Input and Removal Treatments (DIRT) plots of Andrews LTER forest to explain the response of the native SOC to increased litter input (Sulzman et al., 2005; Crow et al., 2009b). Epigeic earthworms are known to increase the incorporation of surface C into the upper A-horizon as POM (Lytle et al., 2015). While in

Table 3

Summary of the final structural equation model showing the unstandardized path coefficients, their standard errors, standardized path coefficients and *P*-values.

Path			Path coefficient	Standard error	Standardized path coefficient	<i>P</i> value
Direct effects						
Fungi:bacteria	←	mAG carbon proportion	-0.003	0.001	-0.404	< 0.001
Fungi:bacteria	←	SC carbon proportion	0.002	0.001	0.479	< 0.001
Fungi:bacteria	←	soil pH	-0.024	0.005	-0.518	< 0.001
Fungi:bacteria	←	Lignin content	0.01	0.003	0.406	< 0.001
MRT of slow pool	←	SFA content	-3.113	0.259	-0.713	< 0.001
MRT of slow pool	←	mAG carbon proportion	0.157	0.057	0.184	0.006
MRT of slow pool	←	Fungi:bacteria	-30.178	8.609	-0.275	< 0.001
MRT of slow pool	←	soil pH	2.125	0.367	0.416	< 0.001
Temperature sensitivity of slow pool	←	SC carbon proportion	-0.011	0.001	-0.687	< 0.001
Temperature sensitivity of slow pool	←	soil pH	0.075	0.014	0.501	< 0.001
Temperature sensitivity of slow pool	←	C/N ratio	-0.036	0.008	-0.402	< 0.001
Temperature sensitivity of active pool	←	Fungi:bacteria	7.901	1.439	0.826	< 0.001
Indirect effects						
Temperature sensitivity of active pool	←	Lignin content	0.081		0.335	0.078
Temperature sensitivity of active pool	←	Soil pH	-0.191		-0.428	0.099

young forests, any leaf litter C freshly incorporated into the soil would have been processed by the relatively abundant endogeic earthworms. During passage through their gut, the newly incorporated leaf C would have a greater potential to associate with minerals or to be incorporated into aggregates, causing subsequent shifts of SOC to the stable pool (Bossuyt et al., 2006; Lytle et al., 2015; Lubbers et al., 2017).

Different from the leaf litter amended soils, the observed suppression of C mineralization after wood amendments (Fig. 1) was consistent with the relatively slower decay rates expected of woody debris and the different incorporation patterns in young and old forests (Lajtha et al., 2005; Lorenz et al., 2007; Preston et al., 2009). In the young forests, lignin from wood amendments was incorporated into POM within microaggregates and associated with silts and clays (SC), which are considered to be more physically and chemically protected (Table S2) (Six et al., 2002; Ma et al., 2014). Thus, in young forests, the incorporated wood C would likely exhibit limited accessibility initially at the incubation experiment, essentially suppressing C mineralization until a later period in the incubation. In old forests, however, where wood C was mostly incorporated into POM fractions with a relatively lower level of protection, the suppression of mineralization from newly incorporated wood C would happen during the earlier stages of incubation (Fig. 1).

Consistent with basidiomycetes being the major decomposers of woody debris in terrestrial environments (Harmon et al., 2004), the ratio of fungi to bacteria (F/B ratio) increased in all wood amended soils (Fig. 4). In addition, the F/B ratio increased more in old forest soils than young forest soils, although the total amount of C and lignin incorporation into surface soils was higher in young than in old forests (Table S2). One possible reason was the lower pH in old forest soils (4.09 vs 5.17 in old and young forest soils respectively) that favored higher basidiomycete activity (Blagodatskaya and Anderson, 1998), which corresponded to the strong effect of soil pH on F/B ratio (Fig. 5). Alternatively, lignin from woody debris may have been incorporated into fractions with higher physical or chemical protection in young forests - either the iPOM fraction or silt and clay (SC) fractions (Fig. S1, Table S2), thus substrates from the incorporated woody debris may have been less accessible to their degraders and less likely to trigger fungal abundance to increase, compared to old forests. The different responses on microbial community triggered by substrate accessibility may further influence SOC stability (Fig. 5).

4.2. Temperature sensitivity of SOC is highly dependent on soil microbial and edaphic properties

No significant interaction between treatment and temperature for either leaf or wood amended C was found in the incubation experiment (Table 2). This lack of interaction is consistent with other litter amendment experiments such as the DIRT studies in Harvard forest, USA and Barbeau National Forest, France, which found a similar temperature sensitivity regardless of the type of aboveground litter manipulation (Boone et al., 1998; Prévost-Bouré et al., 2010). Bosatta and Ågren (1999) demonstrated theoretically that the decomposition of a complex substrate requires higher total activation energy, and so would be more sensitive to rising temperatures than the decomposition of a simple carbon substrate, so-called carbon-quality-temperature theory (CQT theory). However, one important prerequisite for the CQT theory to be true is that chemistry or thermodynamics are the only controlling factors for temperature sensitivity of C mineralization. Usually this is not the case, especially in the complex soil matrix, where carbon substrate availability and additional environmental factors may be significant drivers of SOC degradation (Conant et al., 2011; Dungait et al., 2012; Hopkins et al., 2014). Indeed, temperature sensitivity of organic compounds from litter addition is heavily influenced by abiotic and biotic environmental factors after they enter the soil system. Although there may be easily accessible fractions of soil, like the active pools, which should respond as the CQT theory predicts, most SOC remains

under environmental and physicochemical constraints that obscure the intrinsic temperature sensitivity of its decomposition by microbes (Fang et al., 2005; Davidson and Janssens, 2006). In agreement with this hypothesis, the SEM modeling results of the five-year litter amendment at SERC showed that the temperature sensitivity of the active/labile C pool was more related to microbial community (i.e. F/B ratio) and litter chemistry (i.e. lignin content) than to environmental factors. Thus, in old forests where newly incorporated wood C was less associated with soil minerals, the temperature sensitivity increased, possibly due to the increased lignin content (Fig. S3). In contrast, the sensitivity of the slow pool was more related to protection by association with silts and clays, and environmental factors like soil pH and nutrient availability (estimated by the soil C/N ratio) (Fig. 5, Table 3).

The SEM results showed that the shift in F/B ratio had a strong effect on the temperature sensitivity of active pool C, indicated by shifts in the microbial community with different C availability at the higher temperature (standardized path coefficient = 0.826; Fig. 5, Table 3). Correspondingly, fungal abundance decreased from 1 to 6 months at the higher incubation temperature for both young and old forest soils (Fig. 2), suggesting either a more rapid depletion of nutrients at 25 °C or a relatively greater temperature sensitivity of fungi above 15 °C (Kaufman et al., 1963; Feng and Simpson, 2009). During the soil sampling and incubation preparation processes, fresh substrates and necromass or DOC addition during the inoculation processes could support high populations of fungi and bacteria (Scheu and Parkinson, 1994). After the initial period of rapid substrate utilization, fungal biomass, which largely depends on available fresh plant litter-derived substrates, decreased quickly, especially under the elevated temperature where substrates were consumed faster and microbes had lower C use efficiency (Rinnan et al., 2007; Frey et al., 2008 & 2013; Schindlbacher et al., 2011). In contrast to fungi, bacterial abundance responded less to the higher incubation temperature, decreasing only at 6M in young forest soils, where SOC was more physically protected by microaggregation and thus less accessible (Ma et al., 2013). In addition to substrate constraints, respiratory thermal acclimation of microbes to elevated temperature – defined as “the subsequent adjustment in the rate of respiration to compensate for an initial change in temperature” (Atkin and Tjoelker, 2003), may also determine the temperature sensitivity of soil respiration. Thermal acclimation of respiration has been demonstrated for both ectomycorrhizal (Malcolm et al., 2008) and arbuscular mycorrhizal fungi in soils (Heinemeyer et al., 2006) and for the fungal symbiont in lichens (Lange and Green, 2005). This acclimation may be a mechanism to protect fungi from either heat stress or limited C/nutrient sources.

4.3. Implications for forest C stabilization and future global change

The results from this study indicated that changes in forest SOC stability under increased litter input rates and temperatures will be highly dependent on forest age where SOC was protected differently according to their physical distribution among aggregates. In this incubation study, five years of wood litter amendments stabilized SOC in both young and old forest soils relative to controls. However, in old forests, the effect was achieved by a decrease in the size of the active C pool, while in young forests the MRT of the slow pool was increased. This finding from a long-term laboratory incubation corresponds to field observations from the site, where in young forests the litter C was incorporated into mineral-associated fractions (Ma et al., 2014), while in old forests, the newly incorporated C was apparent predominantly as particulate organic matter. Although no significant change was observed in SOC content, increased leaf litter input also had a significant effect in stabilizing SOC, with the exact effect differing with forest age. The spatial soil heterogeneity observed at SERC forests may also partially explain the varied response of SOC pools to litter addition in other DIRT sites (Crow et al., 2009b; Lajtha et al., 2014; Pisani et al., 2016; Prévost-Bouré et al., 2010; Sulzman et al., 2005;).

These different responses of SOC pools to aboveground litter amendments may also be attributed to the distinct ecological behavior of different earthworm species that dominate SERC young and old forests. It is important to note that while earthworm communities show distinct patterns among forest stands, they are not in steady state locally (Szalavecz et al., 2018). Moreover, abundance and community composition do not follow directional changes based on traditional vegetation succession and forest floor development theory (Pizl, 1992; Trap et al., 2011). Thus, along with earthworm community changes, altered aboveground litter input and climatic conditions, the response of SOC pools and subsequent C stabilization processes may also lead to new trajectories in the future.

In addition to SOC pool sizes and MRTs, the SEM model results also indicated the importance of SOC protection mechanisms, especially the association with silts and clays, soil pH, and microbial mediations, rather than biochemical recalcitrance in controlling temperature sensitivity of SOC dynamics especially for slower cycling C pools. Thus, soil C cycling models that explicitly incorporate factors that influences the protection and accessibility of C as well as microbial acclimation in the soil matrix should improve prediction of SOC dynamics in response to enhanced aboveground litter input and elevated temperature.

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Appendix A. Supplementary data

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